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Polychlorinated biphenyls induce caspase-dependent cell death in cultured embryonic rat hippocampal but not cortical neurons via activation of the ryanodine receptor

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Abstract

Perinatal exposure to polychlorinated biphenyls (PCBs) is linked to cognitive deficits in humans and experimental animals; however, the mechanism(s) underlying this effect remain speculative. Apoptosis is essential to normal brain development, and perturbation of normal spatiotemporal patterns of apoptosis can cause persistent neural deficits. We tested the hypothesis that PCBs alter apoptosis in neuronal cell types critical to cognitive function. Primary cultures of rat cortical and hippocampal neurons were treated for 48 h with Aroclor 1254 or the congeners PCB 77 and 47, which represent coplanar and noncoplanar PCBs that bind the arylhydrocarbon receptor (AhR) with high and low affinity, respectively. Using Hoechst dye and an ELISA for DNA oligonucleosomes, we observed that Aroclor 1254 (10 μM) and PCB 47 (1 μM) significantly increased DNA fragmentation in hippocampal but not cortical neurons, and this effect was blocked by the caspase inhibitors, z-VAD-*fmk* and DEVD-CHO. In contrast, PCB 77 had no effect on apoptosis in either neuronal cell type, suggesting that PCB-induced apoptosis occurs independent of the AhR. The proapoptotic activity of PCBs was inhibited by the ryanodine receptor (RyR) antagonist FLA 365 and by the antioxidant α-tocopherol but not by antagonists of the IP_3 receptor (xestospongin C), L-type calcium channel (verapamil), or NMDA receptor (APV). These data indicate that noncoplanar PCBs induce apoptosis in hippocampal neurons subsequent to RyR activation and increased reactive oxygen species and suggest that altered regional profiles of apoptosis may be an important mechanism underlying the developmental neurotoxicity of PCBs.

Keywords: PCBs; Apoptosis; Hippocampal neurons; Ryanodine receptor; Reactive oxygen species; Vitamin E; Developmental neurotoxicity

Introduction

Polychlorinated biphenyls (PCBs) are a structurally related group of persistent, highly lipophilic chemicals with widespread distribution in the environment (Safe, 1994). Currently, much attention is focused on the potential for environmental levels of PCBs to adversely impact the developing nervous system (USEPA, 1998; NIEHS, 1999; Schmidt, 1999). Epidemiological data suggest that perinatal PCB exposure is associated with decreased IQ scores, impaired learning and memory, sensorimotor dysfunction, and attentional deficits in children (Seegal, 1996; Weisglas-Kuperus, 1998; Ribas-Fito et al., 2001). Similarly, behavioral studies in nonhuman primates and in rodents indicate that developmental exposure to PCBs can cause persistent cognitive deficits (Tilson et al., 1990; Tilson and Kodavanti, 1998). However, assessing the risk posed by PCBs to the developing nervous system is complicated by a general lack of information regarding the mechanism(s) of PCB developmental neurotoxicity. Overt pathology and persistent neurochemical changes are not seen in the brains of PCB-

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exposed humans and animals (Brouwer et al., 1999), thus a predominant theory is that PCB-induced cognitive deficits reflect subtle organizational defects of the brain (Seegal, 1996; Gilbert et al., 2000). At the molecular level, PCBs increase intracellular levels of calcium (Ca^{2+}) and reactive oxygen species (ROS) in cultured neurons, suggesting that PCBs modulate signaling pathways critical to brain development (Seegal, 1996; Tilson and Kodavanti, 1998; Voie and Fonnum, 2000; Mariussen et al., 2002).

A molecular component that may be common to PCB effects on Ca^{2+} and ROS signaling is the ryanodine receptor (RyR), a Ca^{2+}-mediated Ca^{2+} channel localized to the endoplasmic reticulum (ER). It has been demonstrated that PCBs activate the RyR causing release of Ca^{2+} from the ER (Wong et al., 1997a), which in turn can increase production of ROS (Ermak and Davies, 2002; Ravagnan et al., 2002). There is also evidence that ROS can directly modulate the channel activity of the RyR (Feng et al., 2000; Pessah, 2001). But demonstrating a cause–effect relationship between PCB activation of RyR, PCB-modified Ca^{2+}/ROS signals, and altered brain development has been challenging, in part because specific neurodevelopmental events disrupted by PCBs have yet to be identified.

One neurodevelopmental event that may be susceptible to modulation by PCBs is apoptosis. Increased Ca^{2+} and ROS are significant triggers, and RyR activation a critical component, of apoptotic signaling pathways (Berridge et al., 2000; Carmody and Cotter, 2001; Robertson et al., 2001; Ermak and Davies, 2002; Ravagnan et al., 2002). Moreover, PCBs have been shown to cause apoptosis in vascular endothelial and immune cells (Shin et al., 2000; Slim et al., 2000; Jeon et al., 2002) and in PC12 cells grown in the absence of nerve growth factor (NGF) (Shin et al., 2002). Apoptosis is essential to normal brain development (Dikranian et al., 2001; Martin, 2001), occurring in proliferative zones and in postmitotic cells in both the fetal and postnatal brain (White and Barone, 2001). The spatiotemporal pattern of apoptosis in the developing CNS is tightly regulated and disruption of either the timing or the magnitude of apoptosis in a given brain region can alter cell number and thus connectivity, causing deficits in higher-order function even in the absence of obvious pathology (Barone et al., 2000; Sastry and Rao, 2000; Martin, 2001).

The goals of the study reported herein were twofold: (1) to test the hypothesis that PCBs induce apoptosis in neuronal cell types critical to cognitive behavior and (2) to determine whether PCB effects on apoptosis are linked to the known molecular actions of PCBs on Ca^{2+} or ROS signals. To address the first goal, apoptosis was quantified in primary cultures of hippocampal and cortical neurons exposed to Aroclor 1254, a commercial PCB mixture whose congener profile is similar to that found in human tissues including breast milk (Hansen, 1999) or to the individual congeners PCB 47 and 77. These congeners were chosen because both are neurotoxic (Hany et al., 1999) and are detected in the brains of rats and monkeys exposed to commercial PCB mixtures (Seegal et al., 1986, 1991a, 1991b). Although they have the same degree of chlorine substitution, PCB 47 (2,2′, 4,4′-tetrachlorobiphenyl) is a noncoplanar PCB with low aryl hydrocarbon receptor (AhR) binding affinity, whereas PCB 77 (3,3′, 4,4′-tetrachlorobiphenyl) is a coplanar PCB with high AhR binding affinity (Safe, 1994). The role of PCB-modified Ca^{2+}/ROS signals in PCB effects on apoptosis was studied by using antioxidants and selective antagonists of Ca^{2+} channels implicated in PCB effects on Ca^{2+} homeostasis. We report that Aroclor 1254 and PCB 47, but not PCB 77, significantly increase apoptosis in hippocampal but not cortical neurons and the proapoptotic activity of PCBs requires activation of the RyR and an increase in ROS.

Materials and methods

Materials. Aroclor 1254 (lot NT01719/TECH purity), PCB 47 (lot NT00023, >98% pure), and PCB 77 (lot NT01448, >99% pure) were purchased from Ultra Scientific (North Kingstown, RI); 2,2′4,6,6′-Pentachlorobiphenyl (PCB 104) was a generous gift from Dr. Stephen Safe (Texas A&M University). Analytical standards of individual PCB congeners (≥97% pure) were obtained from AccuStandard (New Haven, CT) and Ultra Scientific. Solvents of ultra-high purity grade were obtained from J.T. Baker (Phillipsburg, NJ). Dr. Isaac Pessah (University of California at Davis) provided FLA 365. Hoechst 33258 and calcein AM were purchased from Molecular Probes (Eugene, OR); z-VAD-*fmk* from Enzyme Systems Products (Livermore, CA); and acetyl-DEVD-CHO from Calbiochem (San Diego, CA). Staurosporine, 2-amino-5-phosphonopentanoic acid (APV), α- and γ-tocopherol, xestospongin C, and verapamil were purchased from Sigma (St. Louis, MO).

PCB congener-specific analysis of Aroclor 1254. Using previously described methods (Greizerstein et al., 1997), the Toxicology Research Center of the State University of New York at Buffalo analyzed the Aroclor 1254 mixture (Ultra-Scientific, lot NT01719) used in these studies by capillary column gas chromatography with electron capture detection (GC/ECD). Aroclor 1254 was diluted in acetone, and congeners 30 and 204 were added as internal standards prior to analysis. An automated liquid sampler was used to introduce samples into a Hewlett Packard 6890 Gas Chromatograph (Palo Alto, CA) equipped with temperature and pressure programming capabilities and a split/splitless injector. Individual PCB congeners were separated using a 60-m SPB-5 fused-silica capillary column (0.25 mm i.d., 0.25-μm film thickness) from Supelco (Bellefonte, PA). The carrier gas was ultra-pure helium at an initial head pressure of 34 psi and constant flow rate of 1.5 ml/min. The detector make-up gas was ultra-pure nitrogen at a flow rate of 60 ml/min. A sample volume of 1.0 μL was injected into the splitless injector, operated in the splitless mode (0.75 min

splitless vent time, 100 mL/min) at a temperature of 260°C. The detector temperature was 310°C. The oven temperature program started at 130°C, programmed to 200°C at 4°C/min, then to 210°C at 1.0°C/min, and finally to 280°C at 2.0°C/min. The final temperature was held for 5 min.

The GC data were acquired and quantified using Perkin-Elmer TurboChrom software. Identification and quantification of the individual congeners were performed by comparison to reference standards. The analytes were identified by their retention times relative to the internal standards, congener 30 for peaks eluting before congener 101 and congener 204 for congener 101 and those eluting thereafter. Calibration curves (second-order polynomial) based upon dilutions of an EPA PCB Congener Calibration Check solution (Ultra Scientific RPC-EPA) were used for quantification of congeners 8, 18, 28, 44, 52, 66, 77, 101, 105, 118, 126, 128, 138, 153, 170, 180, 187, 195, and 206. The concentrations of congeners not present in the EPA calibration standard were calculated using average response factors, determined from individual congener reference standards. The concentrations of coeluting congeners were calculated using an adjusted response factor based upon the fraction of the individual congeners, using published values for Aroclor congener distributions (Frame et al., 1996; Hansen, 1999). The weight percent distribution of individual congeners was calculated as a percentage of the total PCBs determined.

Tissue culture. Primary cultures of cortical or hippocampal neurons were prepared from embryonic Holtzman rat pups (E18) according to the method of Bading and Greenberg (1991) as modified by Goslin et al. (1998). Briefly, cerebral cortices and hippocampi were dissociated by treatment with trypsin (2 mg/ml) and deoxyribonuclease (0.6 mg/ml) for 2 min at 37°C then exposed to soybean trypsin inhibitor (1 mg/ml) for 10 min on ice. Cells were then resuspended in Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen) as described in Brewer et al. (1993) and plated at a density of 3×10^5 cells per well into 12-well tissue culture plastic or onto 18-mm glass coverslips precoated with poly-D-lysine (100 μg/ml) and laminin (10 μg/ml). Experimental studies were initiated after 5 days in vitro. Under these culture conditions, immunocytochemical analyses indicated that >95% of the cell population was immunoreactive for neurofilament, a neuronal marker, and <5% of the cell population was immunoreactive for the astrocytic marker glial fibrillary acidic protein (GFAP).

PCB treatment of primary neural cultures. Stocks of Aroclor 1254 and the individual congeners (PCBs 47, 77, and 104) were dissolved in 100% DMSO with sonication. The molarity of Aroclor 1254 solutions was determined using a molecular weight of 327 g/mol, which represents the average molecular weight of the mixture (Ultra Scientific). Experiments were initiated in cultured cells by exchanging the existing medium for freshly prepared medium containing PCBs (diluted 1:1000) and/or a pharmacological agent at its final concentration. In experiments involving xestospongin C, neuronal cultures were treated for 40 min with this agent and then washed once prior to adding medium supplemented with PCBs. During these procedures, less than 5% of the cells were lost due to poor adherence to the culture dish.

Cell viability assay. Following a 48-h treatment with PCBs, cells were incubated in calcein AM (0.25 μM, Molecular Probes) in Neurobasal media for 1 h at 37°C. Cells were then incubated with propidium iodide (6.3 ng/μl, Sigma) in L15 medium for 15 min at 37°C. Live cells enzymatically cleave membrane-permeant calcein AM to yield cytoplasmic green fluorescence; in contrast, nonviable cells take up the membrane impermeant dye propidium iodide, which binds nucleic acids to yield nuclear red fluorescence (Vaughan et al., 1995). The number of viable and nonviable cells was visualized in six randomly chosen fields (at 100×) per experimental condition using a Nikon Eclipse E400 microscope equipped with epifluorescence and digital images captured with a Nikon DXM1200 digital camera were quantified using MetaMorph imaging software (Universal Imaging Corp., Downingtown, PA). Data are expressed as the percentage of viable cells per field (mean ± SEM). This experiment was performed in duplicate over three different dissections and comparable results were obtained from each data set.

Analysis of apoptosis by Hoechst 33258. Following treatment with PCBs, cells were fixed in 4% paraformaldehyde in 0.2 M phosphate buffer for 15 min at room temperature, washed with PBS, and incubated with Hoechst 33258 (10 μg/ml in PBS) for 15 min at room temperature. The cells were then washed with PBS and mounted in Elvanol. Fluorescent images at a magnification of 400× were captured with a Nikon DXM1200 digital camera and then quantified by an observer blind to the experimental conditions using MetaMorph imaging software. The number of neurons with intact ñuclei vs the number of neurons with fragmented nuclei was scored in five randomly chosen microscopic fields per culture (each field typically contained between 25 and 50 neurons). The percentage of neurons with fragmented DNA was calculated for each culture as the number of nuclei with fragmented DNA divided by the total number of nuclei multiplied by 100. Two cultures per dissection from three to four different dissections was analyzed per experimental condition ($n = 6$ to 8) with comparable results obtained from each dissection as determined by homogeneity of variance analyses. A second individual blinded to the experimental conditions independently confirmed results in a randomly chosen subset of cultures.

Cell death ELISA. The ELISA sold under the trade name Cell Death Detection ELISA^{PLUS} (Roche Diagnostics, Indianapolis, IN) was used according to the manufacturer's

instructions to quantify DNA/histone mono- and oligonucleosomes in cultured hippocampal neurons (3×10^5 cells per well in 12-well plates) following exposure to PCBs. Each experimental condition was run in triplicate and data are expressed as a percentage of control (vehicle-treated) values. These experiments were repeated in cultures from three different dissections with comparable results obtained from each dissection.

Statistical analysis. Normally distributed data were analyzed using a one-way ANOVA with Fisher's least significant difference as a post hoc test (Sigma Stat 2.0, SPSS). Nonparametric data were analyzed using ANOVA on ranks followed by Dunnett's post hoc test. Student's *t* test was used for individual comparisons as appropriate. Statistical probability of $p < 0.05$ was considered significant. Data are expressed as the mean ± SEM.

Results

Congener-specific analysis of Aroclor 1254

Aroclor 1254 is a commercial mixture of PCBs defined as being 54% chlorine by weight; however, the congener profile can vary considerably from lot to lot with respect to not only the specific congeners present, but also the amount of any given congener as determined by percent weight distribution (Frame et al., 1996). It has been recently reported that different lots of Aroclor 1254 exert very different biological effects in both whole animal and in vitro model systems (Burgin et al., 2001; Kodavanti et al., 2001). Therefore, to facilitate comparisons across studies, it is critical to determine the composition of Aroclor 1254 used in each investigation with respect to the specific congeners present in the mix and their percent weight distribution. Since this information was not available from the supplier (Ultra Scientific), we determined the congener profile of the Aroclor 1254 mixture (lot NT01719) used in our bioassays by GC/ECD and the results of these analyses are shown in Table 1. These data indicate that this lot of Aroclor 1254 is most similar to Lot G (Frame et al., 1996) and consisted predominantly (>97% by weight) of congeners with four or more chlorine substitutions of which >69% by weight had two or more *ortho* substitutions.

Effects of PCBs on the viability of cultured cortical and hippocampal neurons

Neurons were dissociated from the cortex or hippocampus of E18 rat pups and maintained in serum-free medium for 5 days prior to the initiation of experiments. Under these culture conditions, cell populations were >95% neuronal as assessed by immunoreactivity for the neuron-specific antigen neurofilament and the glia-specific antigen GFAP (data not shown). Cultures were exposed to either the commercial PCB mixture Aroclor 1254 or to individual PCB congeners that had been dissolved in DMSO and then diluted 1:1000 directly into tissue culture medium. Control cultures were treated with medium alone or exposed to medium containing the vehicle, DMSO, diluted 1:1000. After a 48-h exposure to PCBs, cell viability was assessed by the uptake of calcein AM and propidium iodide (Vaughan et al., 1995). The viability of cortical neurons was decreased, but not significantly so, by the highest concentration of Aroclor 1254 tested (30 μM) and was not altered by either PCB 47 or 77 at concentrations up to 10 μM (Fig. 1A and C). In contrast, Aroclor 1254 and PCB 47 significantly decreased the viability of hippocampal neurons at concentrations ≥20 or 3 μM, respectively (Fig. 1B and D). At these higher concentrations, PCB toxicity was manifest morphologically as neurite fragmentation and regression and shrinking or lysis of the soma (data not shown). PCB 77 had no significant effect on viability in hippocampal neurons over the concentration range tested (Fig. 1D). Similar results were obtained after a 24-h exposure to the same concentration range (data not shown).

PCBs induce apoptosis in hippocampal but not cortical neurons

It has been reported that PCBs induce apoptosis in non-neuronal cells at concentrations lower than those that cause a generalized decrease in cell viability (Slim et al., 2000; Jeon et al., 2002). Thus, we examined the effects of PCBs on levels of neuronal apoptosis following a 24- or 48-h exposure to concentrations of Aroclor 1254, PCB 47, or PCB 77 that were observed to cause minimal to no cytotoxicity in prior viability studies. A subset of neuronal cultures was exposed to staurosporine, which is known to induce apoptosis in neuronal cells via activation of caspases (Krohn et al., 1998). To identify apoptotic cells, cultures were stained with the Hoechst 33258 dye and assessed for condensation and fragmentation of nuclear chromatin using epifluorescence microscopy. Staurosporine induces condensation and fragmentation of nuclear DNA in both cortical and hippocampal neurons (Fig. 2). A 24-h exposure to Aroclor 1254 to concentrations ≤30 μM had no effect on the nuclear morphology of either neuronal cell type (data not shown). However, after a 48-h exposure, Aroclor 1254 induced changes in the nuclei of hippocampal neurons comparable to those observed in staurosporine-exposed cultures but had no effect on the nuclear morphology of cortical neurons (Fig. 2). Effects of Aroclor 1254 on apoptosis in hippocampal neurons following a 48-h exposure are statistically significant at 10 μM, causing a 300% increase in the percentage of hippocampal neurons actively undergoing apoptosis relative to vehicle control cultures (Fig. 3). This is in marked contrast to cortical neurons, which exhibit no increase in apoptosis following exposure to the same concentrations of Aroclor over the same time period (Fig. 3).

As determined by GC/ECD analysis, the Aroclor 1254

Table 1
Congener-specific analysis of Aroclor 1254 (Lot NT01719)

IUPAC No.	PCB structure	*o*-CI	Percent composition	IUPAC No.	PCB structure	*o*-CI	Percent composition
4 + 10	2,2′ + 2,6	2 + 2	0.04	118	2,3′,4,4′,5	1	6.8
5 + 8	2,3 + 2,4′	1 + 1	0.41	123	2,3′,4,4′,5	1	0.38
6	2,3′	1	0.04	126 + 129	3,3′,4,4′,5 + 2,2′,3,3′,4,5	0 + 2	0.66
7 + 9	2,4 + 2,5	1 + 1	0.03	128	2,2′,3,3′,4,4′	2	1.46
15 + 17	4,4′ + 2,2′,4	0 + 2	0.06	132	2,2′,3,3′,4,6′	3	2.58
16 + 32	2,2′,3 + 2,4′,6	2 + 2	0.11	134	2,2′,3,3′,5,6	3	0.44
18	2,2′,5	2	0.15	135	2,2′,3,3′,5,6′	3	1.06
19	2,2′,6	3	0	136	2,2′,3,3′,6,6′	4	1.17
22	2,3,4′	1	0.14	138 + 163	2,2′,3,4,4′,5′ + 2,3,3′,4′,5,6	2 + 2	6.84
24 + 27	2,3,6 + 2,3′,6	2 + 2	0.01	141	2,2′,3,4,5,5′	2	1.19
25	2,3′,4	1	0	142	2,2′,3,4,5,6	3	0.24
28	2,4,4′	1	0.14	147	2,2′,3,4′,5,6	3	0.18
31	2,4′,5	1	0.28	149	2,2′,3,4′,5′,6	3	5.26
33	2′,3,4	1	0.24	151	2,2′,3,5,5′,6	3	1.55
40	2,2′,3,3′	2	0.19	153	2,2′,4,4′,5,5′	2	4.64
42	2,2′,3,4′	2	0.2	156 + 171	2,3,3′,4,4′,5 + 2,2′,3,3′,4,4′,6	1 + 3	1.1
44	2,2′,3,5′	2	2.1	157 + 200	2,3,3′,4,4′,5′ + 2,2′,3,3′,4,5,6,6′	1 + 4	0.17
45	2,2′,3,6	3	0.09	167	2,3′,4,4′,5,5′	1	0.29
46	2,2′,3,6′	3	0.02	169	3,3′,4,4′,5,5′	0	0
47	2,2′,4,4′	2	0.1	170	2,2′,3,3′,4,4′,5	2	0.63
48	2,2′,4,5	2	0.04	172	2,2′,3,3′,4,5,5′	2	0.09
49	2,2′,4,5′	2	1.38	174	2,2′,3,3′,4,5,6′	3	0.59
52	2,2′,5,5′	2	5.1	176	2,2′3,3′,4,6,6′	4	0.69
55	2,3,3′,4	1	0.93	177	2,2′,3,3′,4′,5,6	3	0.38
59	2,3,3′,6	2	0.01	179	2,2′,3,3′,5,6,6′	4	0.26
60	2,3,4,4′	1	0.34	180	2,2′,3,4,4′,5,5′	2	0.92
64	2,3,4′,6	2	0.75	181	2,2′,3,4,4′,5,6	3	0
66 + 95	2,3′,4,4′ + 2,2′,3,5′,6	1 + 3	7.5	183	2,2′,3,4,4′,5′,6	3	0.44
70	2,3′,4′,5	1	3.07	185	2,2′,3,4,5,5′,6	3	0.05
74	2,4,4′,5′	1	0.51	187	2,2′,3,4′,5,5′,6	3	0.35
77 + 110	3,3′,4,4′ + 2,3,3′,4′,6	0 + 2	10.64	188	2,2′,3,4′,5,6,6′	4	1.18
82	2,2′,3,3′,4	2	1.07	189	2,3,3′,4,4′,5,5′	1	0.02
87	2,2′,3,4,5′	2	3.93	190	2,3,3′,4,4′,5,6	2	0.04
94	2,2′,3,5,6′	3	0	194	2,2′,3,3′,4,4′,5,5′	2	0.03
97	2,2′,3′,4,5	2	3.16	195	2,2′,3,3′,4,4′,5,6	3	0.01
99	2,2′,4,4′,5	2	3.24	196 + 203	2,2′,3,3′,4,4′,5,6′ + 2,2′,3,4,4′,5,5′,6	3 + 3	0.06
101	2,2′,4,5,5′	2	9.17	205	2,3,3′,4,4′,5,5′,6	2	0
105	2,3,3′,4,4′	1	2.84	206	2,2′,3,3′,4,4′,5,5′,6	3	0

used in our studies contains both coplanar and noncoplanar PCB congeners. Although noncoplanar congeners predominate as determined by percent weight distribution, the presence of coplanar congeners raises the question of whether the proapoptotic activity of Aroclor 1254 is mediated by the AhR. To explore this question, we quantified DNA fragmentation in neurons exposed for 24 or 48 h to varying concentrations of PCB 77, a coplanar congener that is a known ligand of AhR, or PCB 47, a noncoplanar *ortho*-substituted congener that does not bind to the AhR (Safe, 1994). Similar to the observations made with Aroclor 1254, a 24 h exposure to either congener at concentrations $\leq$10 μM had no effect on the nuclear morphology of either hippocampal or cortical neurons. However, following a 48-h exposure, PCB 47 at 1 μM increased the percentage of hippocampal neurons with fragmented DNA by 500% relative to levels measured in control cultures exposed to DMSO but had no effect on cultured cortical neurons (Figs. 2 and 4). Exposure for 48 h to similar concentrations of PCB 77 ($\leq$1 μM) had no effect on apoptosis in either neuronal cell type (Figs. 2 and 4). These morphometric assays of apoptosis were confirmed using an ELISA that quantifies oligonucleosomal DNA generated by cleavage of nuclear DNA in apoptotic cells. ELISA data similarly indicated that Aroclor 1254 (data not shown) and PCB 47 but not PCB 77 significantly increases apoptosis in cultured hippocampal neurons relative to DMSO controls (Fig. 4C).

Although DNA fragmentation is a hallmark feature of apoptotic cell death, it can be observed in other forms of cell death (Olney et al., 2000). Thus, to determine whether PCB-induced changes in nuclear morphology resulted from apoptosis, we quantified the effects of specific inhibitors of caspase activity on PCB-induced DNA fragmentation and condensation. The pan-caspase inhibitor z-VAD-*fmk* at 60 μM (Hamabe et al., 2000) not only effectively blocked DNA fragmentation in hippocampal neurons treated with

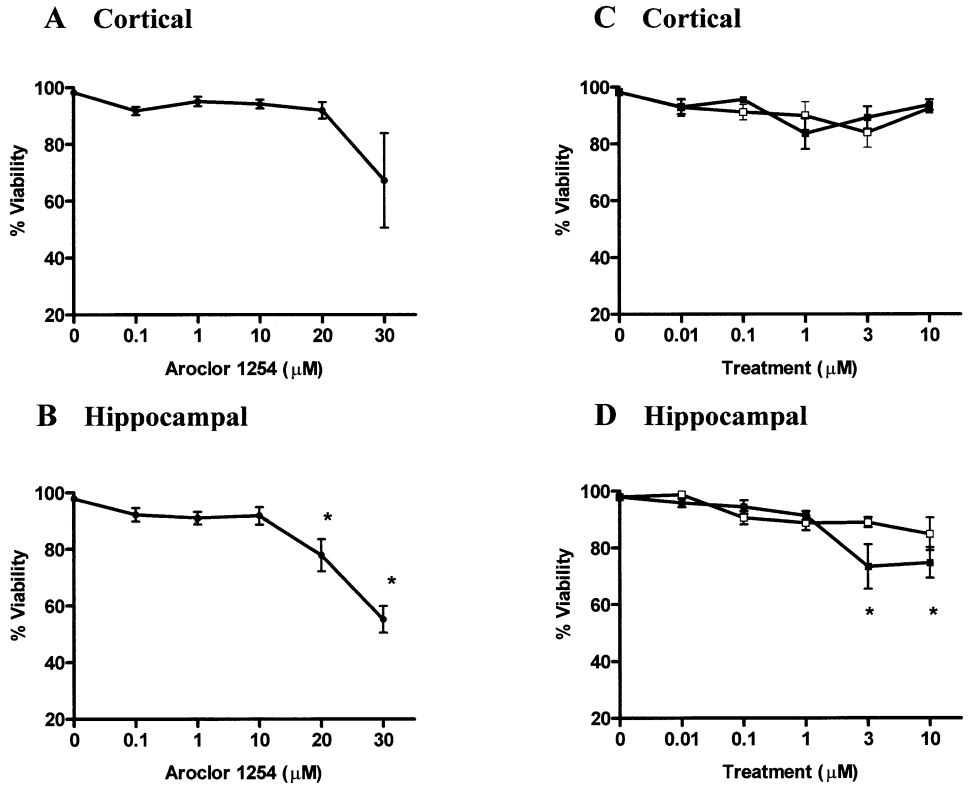

Fig. 1. Hippocampal neurons are more sensitive than cortical neurons to the cytotoxic effects of PCBs. The viability of cortical (A and C) and hippocampal (B and D) neurons treated for 48 h with either Aroclor 1254 (A and B) or purified congeners PCB 47 (closed boxes) or PCB 77 (open boxes) (C and D) was determined using the Molecular Probes live/dead assay. A decrease in cortical neuron viability, which was not statistically significant, was evident only at the highest tested concentration of Aroclor 1254 (30 μM). In contrast, viability in hippocampal neurons was significantly decreased at 20–30 μM Aroclor 1254 and 3–10 μM PCB 47. Values are expressed as percent viability (mean ± SEM). *Significantly different from vehicle control by ANOVA ($p < 0.01$).

staurosporine, but also reduced the percentage of neurons exhibiting apoptotic nuclear morphology in hippocampal cultures exposed to Aroclor 1254 (10 μM) or PCB 47 (1 μM) to levels observed in vehicle control cultures (Fig. 5). Cotreatment with DEVD-CHO (60 μM), a relatively specific inhibitor of caspase-3 (Hamabe et al., 2000), also completely blocked apoptosis in response to Aroclor 1254 or PCB 47 (Fig. 5).

PCB-induced apoptosis is inhibited by a RyR antagonist and by the antioxidant α-tocopherol

It has been previously demonstrated that *ortho*-substituted noncoplanar PCBs, but not coplanar PCBs, increase intracellular calcium (Ca^{2+}) in cultured neurons via a number of mechanisms, including influx of extracellular Ca^{2+} through L-type voltage-sensitive Ca^{2+} channels or the NMDA receptor (Mundy et al., 1999; Inglefield and Shafer, 2000) or release of intracellular Ca^{2+} stores subsequent to activation of RyR (Wong et al., 1997a) or IP_3 receptors (Inglefield et al., 2001). Since increased intracellular Ca^{2+} has been linked to apoptosis in a number of cell types (Berridge et al., 2000; Ermak and Davies, 2002), we investigated the role of these various Ca^{2+} channels in PCB-induced apoptosis using channel-specific pharmacological antagonists. To block Ca^{2+} influx across the plasma membrane, cultured hippocampal neurons were simultaneously exposed for 48 h to PCBs and either APV (100 μM), an antagonist of the NMDA channel, or verapamil (30 μM), an L-type voltage-sensitive Ca^{2+} channel blocker. These concentrations of APV (Lee et al., 1998) and verapamil (Keith et al., 1994; Kodavanti et al., 1994) have previously been shown to block Ca^{2+} influx in primary neuronal cultures. In cultured hippocampal neurons, APV itself caused a significant increase in apoptosis relative to that observed in vehicle control cultures, but less than that induced by PCB 47, whereas verapamil in the absence of PCBs had no significant effect on the percentage of apoptotic cells. Neither antagonist altered PCB 47-induced apoptosis in hippocampal neurons (Fig. 6).

The role of the IP_3 receptor in PCB-induced apoptosis was tested by pretreating cultured hippocampal neurons with xestospongin C (1 μM) for 40 min (Gafni et al., 1997; Inglefield et al., 2001) prior to exposing cells to PCB 47 (1 μM) for 48 h. The percentage of neurons with DNA fragmentation in cultures pretreated with xestospongin C prior to PCB exposure cultures was comparable to that observed in cultures exposed to PCB 47 in the absence of the IP_3

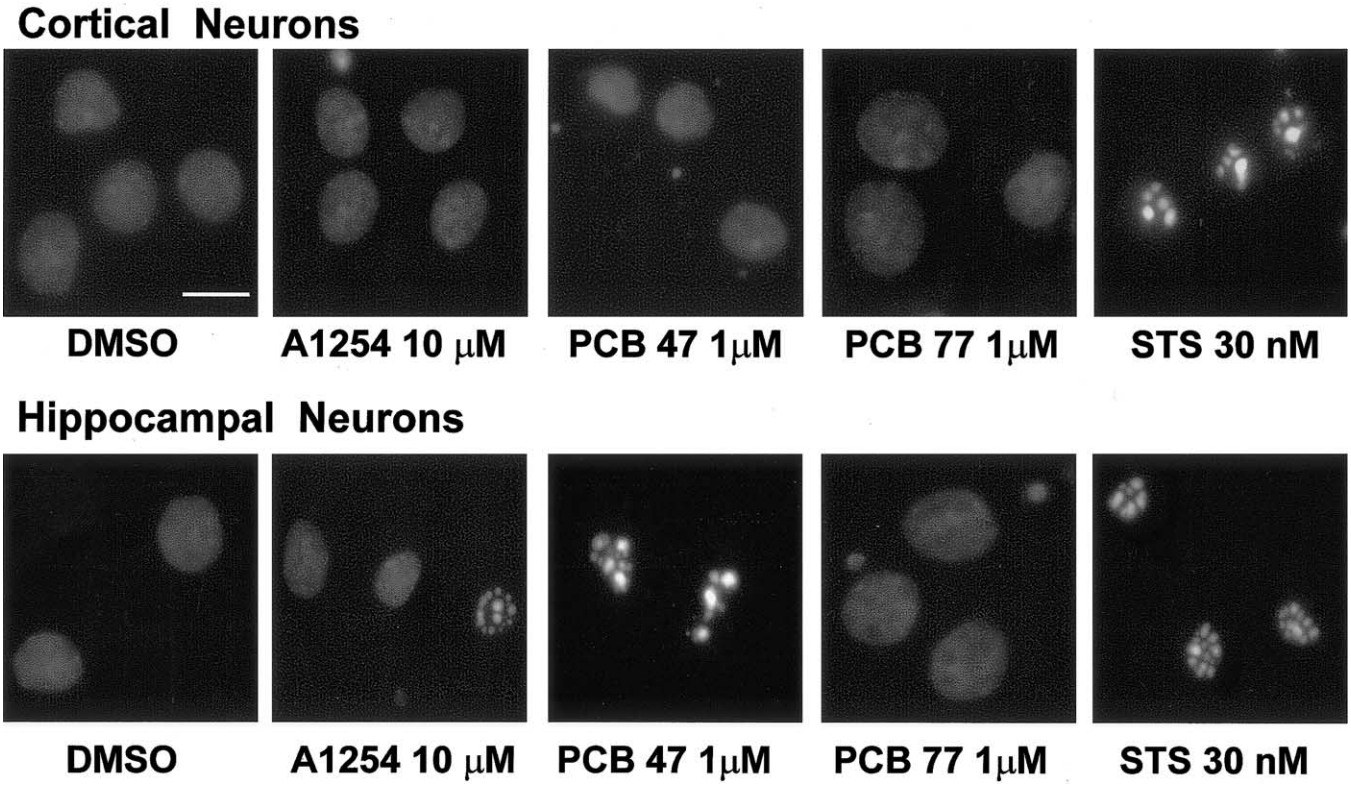

Fig. 2. PCBs induce apoptotic-like nuclear fragmentation in hippocampal but not cortical neurons. Neurons were treated with varying concentrations of PCBs for 48 h or with staurosporine (STS), an agent known to induce apoptosis, for 24 h and stained with Hoechst 33258. Cortical neurons exhibited fragmented DNA only with staurosporine treatment. In contrast, fragmented DNA was observed in hippocampal neurons treated with Aroclor 1254, PCB 47, and staurosporine. Bar represents 20 μm.

receptor antagonist (Fig. 7). In contrast, FLA 365 (1–10 μM), a specific RyR antagonist (Chiesi et al., 1988; Mack et al., 1992), reduced apoptosis in PCB-exposed cultures to levels observed in vehicle control cultures (Fig. 8). Preliminary studies indicated that rapamycin, a pharmacological agent that disrupts the interaction of the immunophilin FKBP12 with RyR and selectively eliminates responses to noncoplanar PCBs (Wong and Pessah, 1997), was cytotoxic to cultured hippocampal neurons following a 48-h exposure to effective concentrations for blocking immunophilin/RyR interactions. Thus, an alternative experimental approach based on previously reported structure–activity relationship (SAR) data from other laboratories was used to verify the role of the RyR in PCB-induced apoptosis. These prior SAR studies indicated that PCB 104, an *ortho*-substituted noncoplanar PCB with five chlorine substitutions, is very active in stimulating translocation of PKC in cultured rat cerebellar granule neurons via calcium influx (Kodavanti et al., 1995; Kodavanti and Tilson, 1997), but does not effectively activate RyRs in cultured cells (Wong and Pessah, 1996). Consistent with the potential role of the RyR in the proapoptotic activity of PCBs, PCB 104 did not increase the percentage of apoptotic neurons in hippocampal or cortical cultures following a 48-h exposure to concentrations ranging from 0.01 to 10 μM (data not shown).

The channel activity of the RyR is regulated by the transmembrane redox potential of the cell (Feng et al., 2000; Pessah, 2001), and increased levels of ROS are a causative factor in at least some types of apoptosis (Carmody and Cotter, 2001; Robertson et al., 2001; Ermak and Davies, 2002; Ravagnan et al., 2002). Since there is evidence that PCBs increase ROS in brain synaptosomes (Voie and Fonnum, 2000) and in cultured cerebellar granule cells (Mariussen et al., 2002), it raised the question of whether increased ROS was an important signal in PCB-induced apoptosis. To address this, we determined whether the antioxidant α-tocopherol protected hippocampal neurons from the proapoptotic activity of PCBs. This vitamin E analog has previously been shown to protect neurons from apoptosis induced by an elevation in intracellular ROS (Krohn et al., 1998), to prevent PCB-mediated cellular damage in vascular endothelial cells (Slim et al., 1999), and to block PCB-induced oxidative stress in cultured cerebellar granule cells (Mariussen et al., 2002). As shown in Fig. 9, cotreatment with α-tocopherol was strikingly effective in reducing DNA fragmentation in cultured hippocampal neurons exposed to PCB 47.

Discussion

Our studies identify apoptosis as a neurodevelopmental event modulated by PCBs and indicate that the mechanism

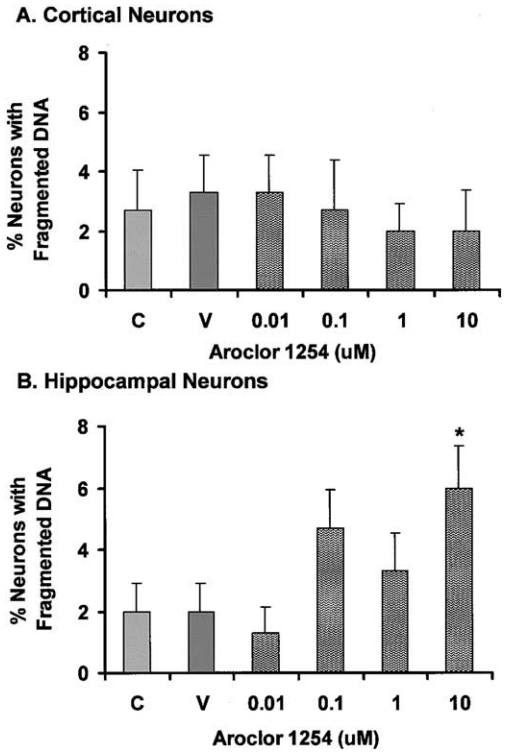

Fig. 3. Aroclor 1254 induces apoptotic-like fragmentation of nuclear chromatin in hippocampal but not cortical neurons. Cortical (A) and hippocampal (B) neurons were treated with varying concentrations of Aroclor 1254 for 48 h and then stained with Hoechst 33258. Negative controls included cultures exposed to medium without vehicle (C) or cultures exposed to medium supplemented with the vehicle DMSO diluted 1:1000 (V). Vehicle had no effect on nuclear morphology in either neuronal cell type. Aroclor 1254 did not alter nuclear morphology in cortical neurons; however, in hippocampal neurons, Aroclor 1254 (10 μM) produced a significant increase in the percentage of neurons with fragmented DNA. Data are expressed as the percentage of neurons with fragmented DNA (mean $\pm$ SEM). *Significantly different from vehicle control by ANOVA ($p < 0.05$).

of PCB-induced apoptosis involves two previously established molecular actions of PCBs: RyR activation and increased production of ROS. The most direct evidence that apoptosis is targeted by PCBs is the observation that Aroclor 1254 and PCB 47 significantly increase apoptosis in primary hippocampal neuron cultures. This finding was confirmed using two diverse methods to detect apoptotic cells: morphometric analysis of nuclear DNA in cells stained with Hoechst dye and quantification of DNA oligonucleosomes by ELISA. PCB induction of neuronal apoptosis was further corroborated by demonstrations that caspase inhibitors attenuate DNA fragmentation in cultures exposed to Aroclor 1254 and PCB 47. Caspases constitute a family of cysteine proteases uniquely associated with apoptosis that catalyze cleavage reactions, resulting in the characteristic morphological changes associated with apoptotic cell death (Alnemri et al., 1996; Cohen, 1997). These enzymes are constitutively expressed in the cytoplasm as proenzymes that must be proteolytically processed to form active enzymes with specific substrate cleavage activity (Martin and Green, 1995). The membrane-permeable pan-caspase inhibitor z-VAD-*fmk* suppresses apoptosis by inhibiting the proteolytic activation of caspases-2, -3, -6, and -7 (Jacobsen et al., 1996; MacFarlane et al., 1997), whereas the selective caspase-3 inhibitor DEVD-CHO prevents sub-

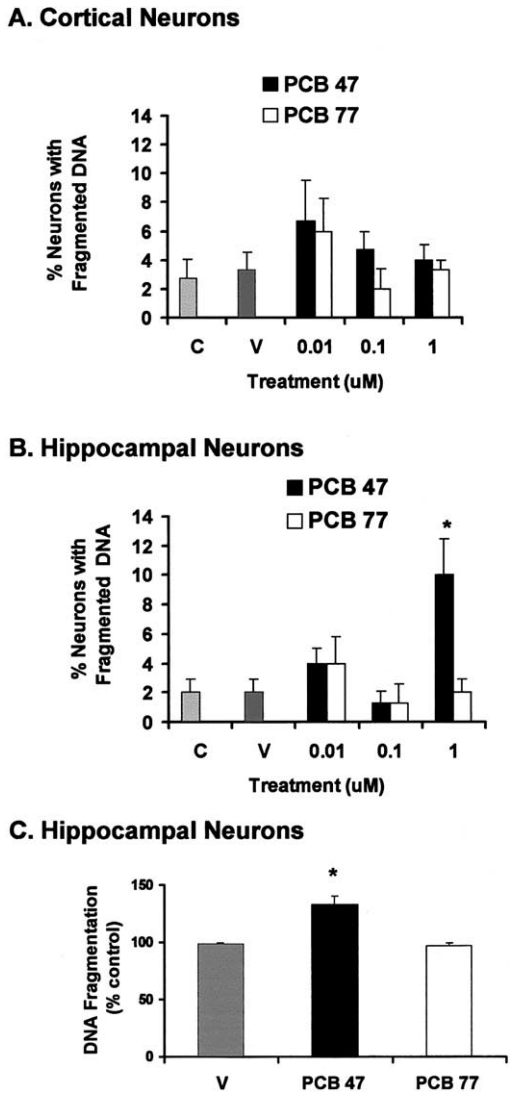

Fig. 4. PCB 47 but not PCB 77 increases apoptotic nuclear morphology in hippocampal but not cortical neurons. Cortical (A) and hippocampal neurons (B) were treated with varying concentrations of PCB 47 or PCB 77 for 48 h and then stained with Hoechst 33258 to visualize nuclear morphology. Neither PCB congener altered the percentage of cortical neurons with fragmented chromatin. In contrast, PCB 47, but not PCB 77 increased the percentage of hippocampal neurons with fragmented DNA. (C) Quantification of apoptosis by "sandwich" ELISA using anti-histone and anti-DNA antibodies similarly indicated that apoptosis is significantly increased in hippocampal neurons exposed to PCB 47 (1 μM) but not PCB 77 (1 μM) for 48 h. ELISA data are absorption values expressed as a percentage of control (absorption values of cultures exposed to DMSO diluted 1:1000). All data are expressed as the mean $\pm$ SEM; C, control cultures exposed to medium alone; V, vehicle control cultures exposed to medium supplemented with DMSO diluted 1:1000. *Significantly different from vehicle control by ANOVA ($p < 0.01$).

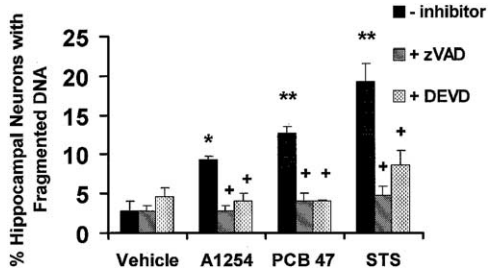

Fig. 5. PCB-induced DNA fragmentation is caspase dependent. Hippocampal neurons were treated with Aroclor 1254 (10 μM) or PCB 47 (1 μM) for 48 h in the presence or absence of the pan-caspase inhibitor z-VAD-*fmk* (60 μM) or the caspase-3 inhibitor DEVD-CHO (60 μM) and then stained with Hoechst 33258. Both zVAD-*fmk* and DEVD-CHO blocked DNA fragmentation induced by staurosporine (30 nM for 24 h), which is known to induce apoptosis via activation of caspases. Similarly both caspase inhibitors prevented DNA fragmentation in neurons treated with PCBs. Data are expressed as the percentage of hippocampal neurons with fragmented DNA (mean ± SEM). *Significantly different from vehicle control by ANOVA ($p < 0.01$), **($p < 0.001$). $^{+}$Significantly different from PCB treatment without zVAD-*fmk* or DEVD-CHO by Student's *t* test ($p < 0.01$).

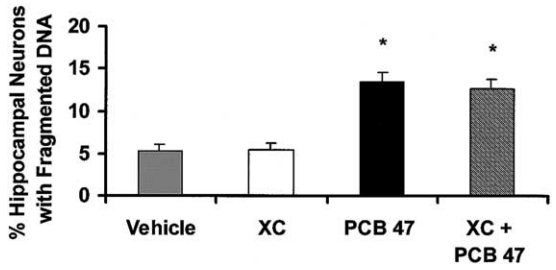

Fig. 7. The IP_3 receptor does not mediate PCB 47-induced apoptosis. Hippocampal neurons were pretreated with the specific IP_3 receptor antagonist xestospongin C (1 μM) for 40 min prior to exposing cells to PCB 47 (1 μM) for 48 h. There was no statistically significant difference between PCB-exposed cells cultured in the presence or absence of xestospongin C. Data are expressed as the percentage of hippocampal neurons with fragmented DNA (mean ± SEM). *Significantly different from vehicle control by ANOVA ($p < 0.001$).

strate binding to the active site of caspase-3 (Swe and Sit, 2000). Treatment with either z-VAD-*fmk* or DEVD-CHO inhibited PCB-induced DNA fragmentation in hippocampal neurons.

The selectivity of PCB-induced apoptosis is suggested by the following observations. First, increased apoptosis was evident following exposure to relatively low concentrations of Aroclor 1254 (10 μM) or PCB 47 (1 μM) that were not cytotoxic. These concentrations are within the range of PCB levels detected in the brains of PN21 rats (2.4 ppm or ~7.2 μM) with behavioral deficits following perinatal exposure to Aroclor 1254 (Crofton et al., 2000). Second, Aroclor 1254 and PCB 47 did not increase apoptosis in cortical neurons derived from the same animals and grown under the same conditions as hippocampal neurons, even though both cell types exhibited increased DNA fragmentation when treated with staurosporine. Similarly, Inglefield et al. (2001) reported that exposure of cultured cortical neurons to Aroclor 1254 (≤20 μM) increased necrotic cell death but did not induce apoptosis as measured by fluorometric analyses of activated caspase-3 and TUNEL labeling. The reason(s) underlying the differential susceptibility of hippocampal and cortical neurons to the proapoptotic effects of PCBs are not known. Possibilities include differential RyR expression (Lai et al., 1992; Sharp et al., 1993; Furuichi et al., 1994; Ledbetter et al., 1994; Giannini et al.,

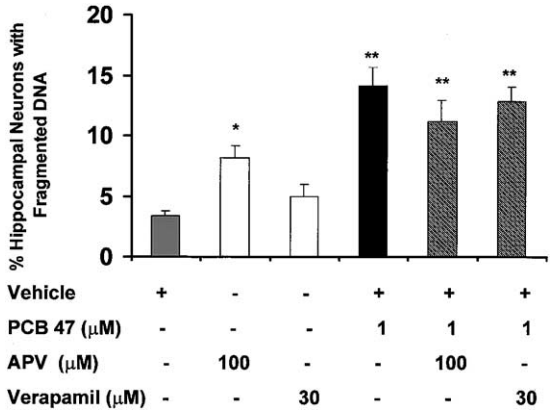

Fig. 6. Extracellular Ca^{2+} is not involved in PCB-induced DNA fragmentation in hippocampal neurons. Hippocampal neurons were treated with PCB 47 (1 μM) for 48 h in the presence or absence of either APV (100 μM), an NMDA channel blocker, or verapamil (30 μM), an L-type Ca^{2+} channel blocker and then stained with Hoechst 33258. There was no statistically significant difference between PCB-exposed cells cultured in the presence or absence of either Ca^{2+} channel blocker. Data are expressed as the percentage of hippocampal neurons with fragmented DNA (mean ± SEM). *Significantly different from vehicle control by ANOVA ($p < 0.01$), **($p < 0.001$).

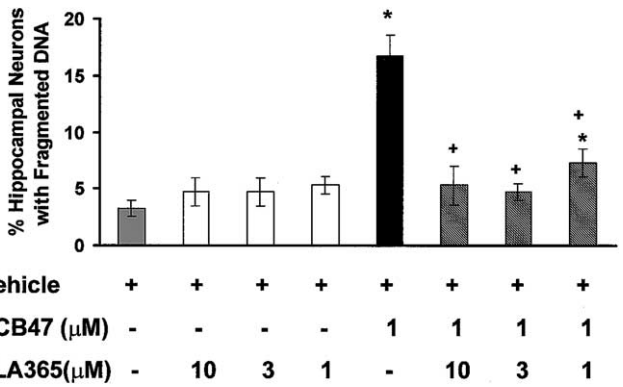

Fig. 8. Blocking the ryanodine receptor prevents PCB 47-induced DNA fragmentation. Hippocampal neurons were treated with PCB 47 (1 μM) for 48 h in the presence or absence of varying concentrations of FLA 365. All tested concentrations of FLA 365 blocked the increase of apoptotic nuclear fragmentation induced by PCB 47. Data are expressed as the percentage of hippocampal neurons with fragmented DNA (mean ± SEM). *Significantly different from vehicle control by ANOVA ($p < 0.05$). $^{+}$Significantly different from PCB treatment without FLA 365 by ANOVA ($p < 0.001$).

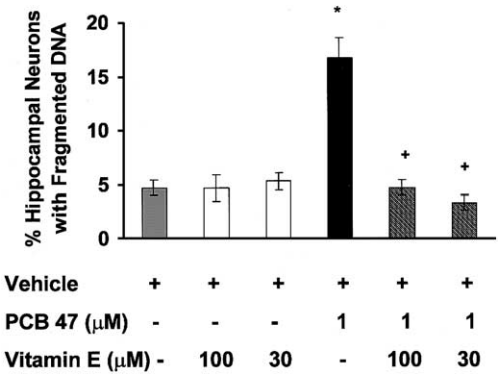

Fig. 9. Vitamin E blocks PCB 47-induced DNA fragmentation. Hippocampal neurons were treated with PCB 47 (1 μM) for 48 h in the presence or absence of α-tocopherol. This analog of vitamin E blocked PCB 47-induced apoptotic DNA fragmentation. Data are expressed as the percentage of hippocampal neurons with fragmented DNA (mean ± SEM). *Significantly different from vehicle control by ANOVA ($p < 0.001$). $^{+}$Significantly different from PCB 47 by ANOVA ($p < 0.001$).

1995) or antioxidant capacity (Wilson, 1997; Homi et al., 2002) between these neuronal cell types. Testing these possibilities is the focus of future research efforts.

A third indication of the selectivity of PCB-induced apoptosis is our finding that proapoptotic activity appears to be restricted to noncoplanar congeners. Consistent with this SAR, preliminary studies indicate that PCB 95, a noncoplanar congener, but not PCB 66, a coplanar congener, exhibits proapoptotic activity in hippocampal neurons (A. Howard and P. Lein, unpublished observations). Although coplanar PCBs have been shown to increase apoptosis in vascular endothelial cells (Slim et al., 2000), the reverse SAR has been reported in studies of PCB-induced apoptosis in monocytic cells (Shin et al., 2000), spleen cells (Jeon et al., 2002), and nontransformed (chromaffin-like) PC12 cells (Shin et al., 2002). These data suggest that, in many cell types, including neurons, PCBs induce apoptosis via an AhR-independent mechanism. More rigorous support of an AhR-independent mechanism includes observations in spleen cells that (1) Aroclor 1254 causes significant apoptosis in the absence of upregulated CYPIA1 mRNA expression or AhR binding to the dioxin response element; (2) the AhR antagonist α-naphthoflavone does not inhibit Aroclor-induced apoptosis; and (3) Aroclor 1254 significantly increases apoptosis in spleen cells from *AhR*-null mice (Jeon et al., 2002). The toxicological relevance of these findings is suggested by reports that noncoplanar PCBs are detected in the brains of humans and animals exposed to complex PCB mixtures and that noncoplanar PCBs cause cognitive deficits subsequent to developmental exposure in animal models (Fischer et al., 1998; Tilson and Kodavanti, 1998).

A similar SAR has been demonstrated for PCB-induced increases in intracellular calcium (Ca^{2+}) mediated by a number of mechanisms, including influx of extracellular Ca^{2+} through L-type voltage-sensitive Ca^{2+} channels or the NMDA receptor (Mundy et al., 1999; Inglefield and Shafer, 2000) or release of intracellular Ca^{2+} stores subsequent to activation of RyR (Wong et al., 1997a) or IP_3 receptors (Inglefield et al., 2001). Noncoplanar PCBs, but not coplanar PCBs, have also been shown to increase ROS in synaptosomes (Voie and Fonnum, 2000) and in cultured neurons (Mariussen et al., 2002). These data, together with the evidence indicating a role for elevated Ca^{2+} and ROS in the activation of apoptotic signaling pathways (Berridge et al., 2000; Carmody and Cotter, 2001; Robertson et al., 2001; Ermak and Davies, 2002; Ravagnan et al., 2002), suggest a link between PCB-modified Ca^{2+}/ROS signaling and apoptosis. More direct evidence of a causal relationship is the inhibition of apoptosis in PCB-exposed hippocampal neurons by FLA 365, a specific RyR antagonist (Chiesi et al., 1988; Mack et al., 1992), or the antioxidant α-tocopherol. Interestingly, antagonists previously shown to block PCB-mediated Ca^{2+} flux through L-type voltage-sensitive channels, NMDA receptors, and IP_3 receptors in cultured neurons did not inhibit PCB-induced apoptosis in our model system, suggesting that proapoptotic activity may be restricted to a subset of PCBs that alter Ca^{2+} signaling via activation of the RyR. The finding that the noncoplanar congener PCB 104 did not induce neuronal apoptosis supports this conclusion since previous in vitro studies have demonstrated that PCB 104 potently stimulates Ca^{2+} influx (Kodavanti and Tilson, 1997) but does not activate the RyR (Wong and Pessah, 1996). These data suggest that the SAR for PCB-induced apoptosis is more complex than just whether a congener is *ortho*-substituted. Similarly, it has been reported that, although cytotoxic effects in cultured cerebellar granule cells are associated only with noncoplanar PCBs, not all noncoplanar PCBs elicit cytotoxicity in this model system (Carpenter et al., 1997).

The hippocampus has long been implicated in learning and memory function in humans and other animals (Squire, 1992). Our observation that PCBs induce apoptosis in cultured hippocampal neurons via RyR activation and increased ROS provides a biologically plausible link between the molecular actions of PCBs and their neurotoxic effects in developmentally exposed humans and experimental animals. In the normal developing rodent hippocampus, apoptosis peaks on postnatal day 1 (White and Barone, 2001), during which it is estimated that 2–3% of cells in the region may be apoptotic at any given time (Bursch et al., 1990). In our model system, PCBs increased the density of apoptotic neurons from a background of 2–5% to levels as high as 18%. This increase may be even more significant if the population of cells undergoing PCB-induced apoptosis represents a localized subset of neurons in the developing hippocampus, for example, CA1 vs CA3 pyramidal neurons. These modest, but significant increases may also reflect differential distribution of RyR within the cell culture population since localization studies have demonstrated that the spatiotemporal distribution of RyRs varies within the hippocampus by subregion, cell type, and developmental stage (Sharp et al., 1993; Giannini et al., 1995). The poten-

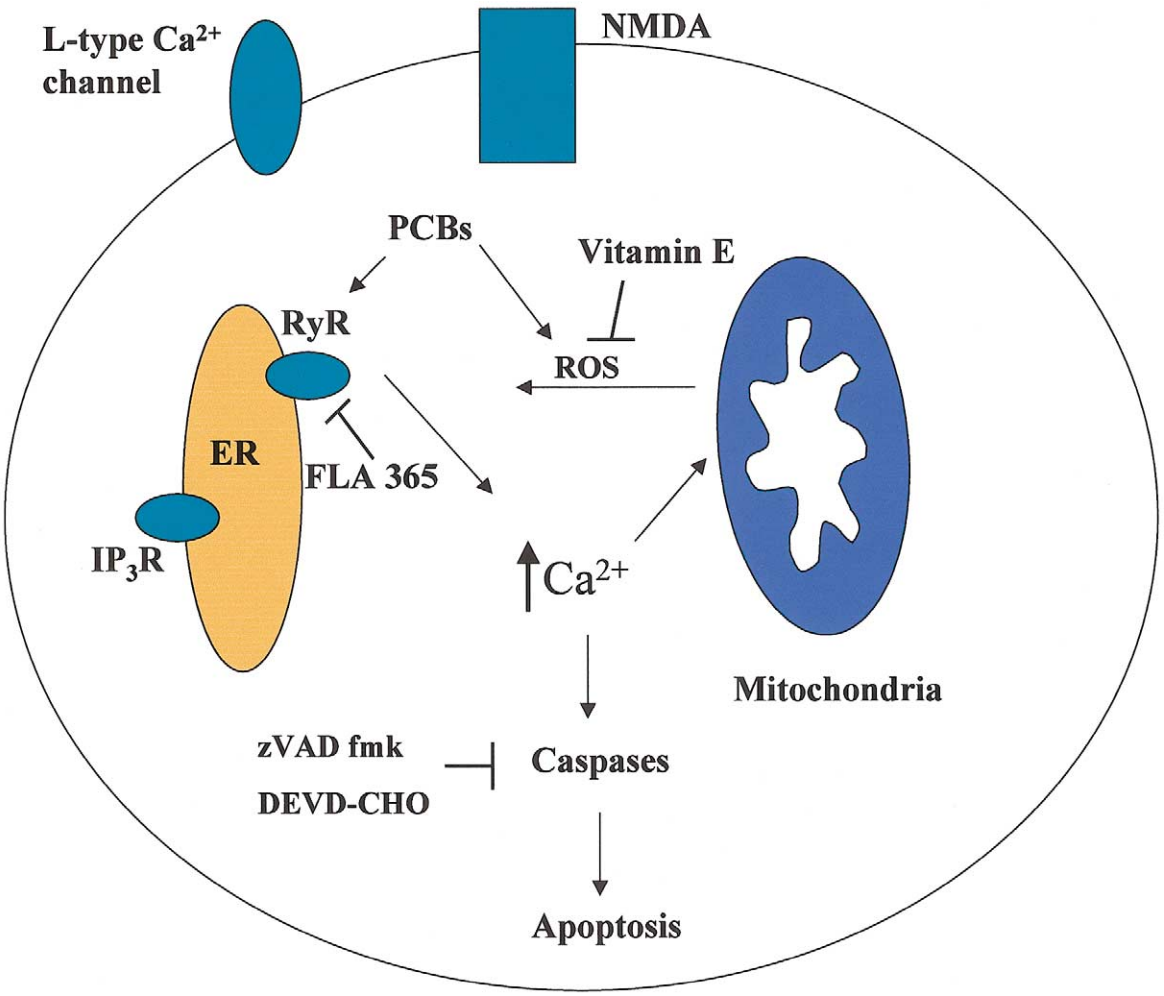

Fig. 10. Postulated mechanisms of PCB 47-induced apoptotic DNA fragmentation. Specific noncoplanar PCBs may directly activate the ryanodine receptor (RyR) causing release of Ca^{2+} from endoplasmic reticular (ER) stores. Increased cytoplasmic Ca^{2+} activates caspases resulting in apoptosis. Increased cytoplasmic Ca^{2+} may also cause increased mitochondrial Ca^{2+} influx, which increases generation of reactive oxygen species (ROS) thereby promoting caspase-dependent apoptosis. Alternatively, PCBs may generate ROS directly, which then increase cytoplasmic levels of Ca^{2+} via activation of RyRs. Blocking the L-type voltage-sensitive Ca^{2+} channel with verapamil or the NMDA receptor with APV does not have any effect on PCB-induced DNA fragmentation, suggesting that, in this model system, extracellular calcium is not involved in the apoptotic signaling pathway.

tial significance of these seemingly modest increases is suggested by observations that exposure of the developing rat brain to concentrations of NMDA antagonists associated with functional deficits increases the density of apoptotic neurons from background levels of 1–2 to 15–26% of the total neuronal density in layer II of parietal, frontal, and cingulate cortices and 12% in the laterodorsal thalamus (Ikonomidou et al., 1999). Similarly, mercury compounds increase the density of apoptotic neurons in embryonic brain cell aggregates from 5 to 15%, which correlates with a significant decrease in the overall size of the aggregate (Monnet-Tschudi, 1998). It is believed that removal of even a small number of postmitotic neurons during synaptogenesis can significantly alter patterns of connectivity (Dikranian et al., 2001; Martin, 2001), resulting in functional deficits in the absence of obvious pathology. This type of neurodevelopmental defect would be consistent with what is known of PCB developmental neurotoxicity. Although it has yet to be demonstrated that PCBs induce apoptosis in the developing hippocampus, it has been shown that perinatal exposure to PCB 95, the most potent PCB activator of the RyR, which also exhibits proapoptotic activity in cultured hippocampal neurons (see above), significantly alters the response of rats in a learning task dependent on hippocampal function (Schantz et al., 1997).

Our data implicate RyR activation and increased ROS in the mechanism(s) underlying PCB-induced apoptosis. The ability of a specific RyR antagonist to block apoptosis in PCB-exposed hippocampal neurons adds to the emerging evidence that activation of the RyR is a critical signaling component in apoptosis (Andjelic et al., 1997; Hajnoczky et al., 2000; Mariot et al., 2000; Pan et al., 2000; Danieli and Rampazzo, 2002). The observation that α-tocopherol also prevents apoptosis in hippocampal neurons exposed to PCB

47 is consistent with evidence that increased ROS can activate apoptotic signaling pathways (Carmody and Cotter, 2001; Robertson et al., 2001; Ermak and Davies, 2002). This latter interpretation is complicated by reports that, in addition to its antioxidant properties, α-tocopherol exhibits other nonantioxidant activities, including membrane stabilization, specific PKC inhibition, and altered gene transcription (Azzi et al., 2000; Azzi and Stocker, 2000). However, two observations suggest that α-tocopherol inhibits apoptosis in PCB-exposed neurons because of its antioxidant properties. First, preliminary data indicates that γ-tocopherol (10 μM) inhibits PCB-induced apoptosis in our model system (P. Lein and A. Howard, unpublished observations) and γ-tocopherol exhibits the antioxidant properties of α-tocopherol (Cooney et al., 1993; Christen et al., 1997; Takahashi et al., 1998) but not its other unique nonantioxidant activities (Azzi et al., 2000; Azzi and Stocker, 2000). Second, it has been demonstrated that PCBs increase ROS in nonneuronal cells (Voie et al., 1998, 2000), brain synaptosomes (Voie and Fonnum, 2000), and cultured cerebellar granule cells (Mariussen et al., 2002). Regardless of the mechanism by which vitamin E analogs inhibit the proapoptotic activity of PCBs, the fact that it does so has broader implications for the development of preventive measures in high-risk populations for PCB developmental neurotoxicity.

How do the RyR and ROS modulate apoptosis in PCB-exposed neurons? Although our data do not directly address this question, when placed in context of evidence in the published literature, at least two plausible models emerge (see Fig. 10). In one model, ROS occurs downstream of RyR activation. Thus, noncoplanar PCBs interact with RyRs in the hippocampus (Schantz et al., 1997; Wong et al., 1997b), which stabilizes the receptor in its open conductance state resulting in greater release of Ca^{2+} from the ER (Wong et al., 1997a). Elevated intracellular Ca^{2+} either directly activates caspases in neuronal cells (Mattson et al., 2000; Ray et al., 2000) and/or increases Ca^{2+} flux into the mitochondria, thereby increasing ROS production, which triggers release of cytochrome *c* from mitochondria with subsequent activation of caspases (Livingstone et al., 2000; Ermak and Davies, 2002; Ravagnan et al., 2002). However, emerging evidence suggests that ROS do not cause apoptotic cell death by passive cumulative oxidative damage, but rather ROS act as signaling molecules that initiate apoptosis via targeted interactions with specific cellular components, one of which is the RyR (Carmody and Cotter, 2001; Robertson et al., 2001; Ermak and Davies, 2002). ROS have been shown to interact directly with hyperreactive cysteine residues on the RyR to enhance its open probability, resulting in release of intracellular Ca^{2+} stores (Suzuki and Ford, 1999; Okabe et al., 2000; Pessah, 2001). These findings suggest an alternative model of PCB-induced apoptotic signaling in which noncoplanar PCBs activate RyR indirectly as a consequence of PCB-induced increase in ROS (Fig. 10). There is precedence for this proposed mechanism in that ROS generated by quinone increase the calcium conductance of RyR from rabbit sarcoplasmic reticulum membranes (Feng et al., 1999). These may not be mutually exclusive models in that PCBs may independently influence both RyR activation and ROS generation, with each effect augmenting the other in a positive feedback mechanism. Further research is required to rigorously test these models and elucidate the relative role(s) of RyR and ROS in the signaling pathway(s) that mediate PCB-induced apoptosis.

In summary, we have demonstrated that PCBs significantly increase apoptosis in hippocampal, but not cortical neurons, via a caspase-dependent but AhR-independent mechanism. Furthermore, the proapoptotic activity of noncoplanar PCBs requires RyR activation and can be prevented by treatment with vitamin E. These data suggest that altered regional profiles of apoptosis may be an important mechanism underlying the developmental neurotoxicity of PCBs.

Acknowledgments

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